

# Discrimination of Hoechst Side Population (SP) Cells in Mouse Bone Marrow with Violet and Near-UV Laser Diodes

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## Abstract

Discrimination of stem cells using flow cytometric analysis of Hoechst 33342 efflux by the ABCG2 transporter (termed the Hoechst "side population", or SP technique) is a valuable methodology for identifying bone marrow progenitor enriched for stem cells. Unfortunately, it requires a UV laser source, usually necessitating an expensive and maintenance-intensive argon- or krypton-ion gas laser on a large-scale cell sorter. We have therefore evaluated the ability of recently available violet and near-UV laser diodes to discriminate Hoechst SP on smaller cuvette-based flow cytometers. Violet laser diodes (emitting at 408 and 401 nm) and near-UV laser diode2 (emitting from 370 to 374 nm) were mounted on a BD Biosciences LSR II and evaluated for their ability to discriminate Hoechst SP in murine bone marrow, in comparison with traditional UV-emitting ion laser on a large-scale stream-in-air cell sorter. The violet laser diodes were able to discriminate the Hoechst SP, but with poorer resolution than the standard UV gas laser on a large-scale cell sorter. The near-UV laser diode, in contrast, gave excellent Hoechst SP resolution. As the next generation of cell sorters integrate cuvette-based cell interrogation into conventional jet-in-air cell separation, these laser source should become applicable for both analysis and physical separation of Hoechst SP cells.

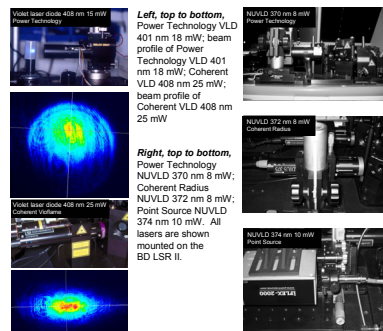
## Materials and Methods

**Mice and cells.** BALB/c female mice four to twelve weeks of age (Jackson Laboratory, Bar Harbor, ME) were maintained in the NIH-NICHD single pathogen free barrier animal colony, and were euthanized immediately prior to bone marrow aspiration according to NIH guidelines. Bone marrow was extracted by fine needle aspiration and washed twice with HBSS containing 2% FBS and 10 mM HEPES. Some bone marrow samples were initially incubated with anti-FcyR2b/3 antibody for ten minutes at 4°C, followed by incubation with PE-conjugated antibodies against the lineage markers B220, Ter-119, CD3, Gr-1 and Mac-1 (BD Pharmingen, San Diego, CA) for twenty minutes at 4°C. Cells were washed and subsequently incubated with anti-PE antibody conjugated paramagnetic beads (Miltenyi Biotec, Auburn, CA) for twenty minutes. Lineage-positive cells were then removed using an AutoMACS cell separation unit (Miltenyi Biotec), using the normal depletion program. The lineage-positive (SP-depleted) and lineage-negative (SP-enriched) populations were washed in the above buffer and counted.

A549 lung carcinoma cells were obtained from the American Red Cross (Rockville, MD) and passaged in Dulbecco's MEM containing 10% FBS. These cells were removed from their growth substrate with trypsin/EDTA, washed with cold HBSS/FBS/HEPES and counted prior to Hoechst 33342 labeling.

**Hoechst 33342 labeling for SP discrimination.** The above cell fractions, unseparated bone marrow and A549 cells were then labeled with Hoechst 33342 using the method previously described by Goodell et al. (1,3). Briefly, cells were resuspended at 10<sup>6</sup> cells per ml in HBSS with 2% FBS and 10 mM HEPES and prewarmed to 37°C. For some samples, bone marrow cells were preincubated with the ABCG2 inhibitor fumitremorgin C at 10 µM for fifteen minutes. Hoechst 33342 was then added at a final concentration of 5 µg/ml and the cells incubated for 90 minutes at 37°C with periodic mixing. Some BM samples were then washed with cold HBSS/FBS/HEPES and simultaneously labeled with FITC-anti-Sca-1 and APC-anti-c-kit. Cells were then washed with and resuspended in HBSS/FCS/HEPES and kept at 4°C until analysis (within four hours).

**Flow cytometry.** Cells were analyzed on one of two instruments: (1) a BD Bioscience FACS Vantage DiVa jet-in-air sorter equipped with argon-ion 488 nm, krypton-ion 408 nm, HeNe 633 nm and one of several violet or near-UV laser diode sources. Hoechst 33342-labeled cells were excited on the FACS Vantage DiVa with a Coherent 1302C krypton-ion laser emitting in multiline UV mode (361 and 367 nm) at 100 mW. Hoechst 33342-labeled cells were excited on the LSR II with one of the following lasers: (i) a Coherent Violette 408 nm violet laser diode emitting at 408 nm; (ii) a Power Technology 401 nm violet laser diode emitting at 408 nm; (iii) a Power Technology 370 nm near-UV laser diode (NUVLD) emitting at 8 mW, (iv) a Coherent 374 nm NUVLD emitting at 8 mW, or (v) a Point Source 374 nm NUVLD emitting at 10 mW. Hoechst blue and red signal were detected with 450/50 nm and 650 longpass filters respectively, split with a 595 longpass dichroic on the LSR II and a 610 SP on the FACS Vantage. Violet, near-UV and UV laser alignments were quality controlled with Molecular Probes InSpect Blue microsphere cocktails. Data was analyzed with WinMDI version 2.8 (Joseph Trotter, BD Biosciences).

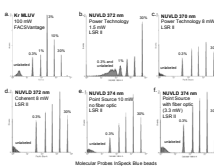


## NUVLD alignment on the BD LSR II

Laser alignment and sensitivity was assessed with Molecular Probes InSpect Blue microsphere cocktails. These mixtures of beads with gradually descending fluorescent intensities allow sensitivity comparisons between laser sources. Sources in the 8 to 10 mW range generally gave comparable sensitivity to much more powerful ion lasers on stream-in-air instruments; this sensitivity declined below 3 mW.

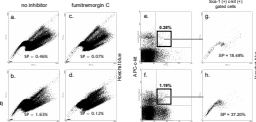
### Alignment and sensitivity check on flow cytometers with UV laser sources.

**a.** FACS Vantage SE with Kr MLUV 100 mW. BD LSR II with **b.** Power Technology NUVLD 372 nm 1.5 mW. **c.** Power Technology NUVLD 370 nm 8 mW. **d.** Coherent NUVLD 372 nm 8 mW. **e.** Point Source NUVLD 374 nm 10 mW. **f.** Point Source laser with single mode fiber (3.3 mW output)



## Hoechst SP analysis on the FACS Vantage SE

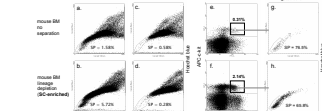
Mouse bone marrow labeled for Hoechst SP was then analyzed on the FACS Vantage SE using the krypton-ion laser in MLUV mode at 100 mW. All samples were analyzed in this fashion as a quality control. Simultaneous analysis of Sca-1 and c-kit (CD117) expression confirmed the identity of the SP cells as stem cell and early progenitors.



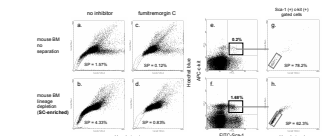
**Above.** Hoechst SP analysis of unpurified (a,c,e,g) and lineage-depleted bone marrow (b,d,f,h) on a FACS Vantage DiVa cell sorter equipped with a krypton-ion laser emitting at MLUV mode. Cells were incubated without (a,b) or with fumitremorgin C (c,d) prior to Hoechst 33342 labeling. The percentage SP-positive cells is indicated for each Hoechst red versus blue cytogram (a,b,c,d). Simultaneous analysis of Sca-1 versus c-kit expression for unpurified and lineage-depleted samples is shown in panels e and f (with percentage Sca-1-positive c-kit-positive cells indicated), and the Hoechst SP population for Sca-1-positive c-kit-positive fractions is shown in panels g and h.

## Hoechst SP analysis with violet laser diodes

While it has been suggested that violet laser diodes (VLD) and other violet lasers might make useful excitation sources for Hoechst SP analysis, comparison with ultraviolet sources (including krypton-ion laser tuned to the violet 407 and 413 nm lines) and particularly VLDs on cuvette instruments like the BD LSR II give much poorer SP resolution. Even high-power violet sources such as krypton-ion lasers (not shown) and low-wavelength VLDs (401 nm below) do not resolve the SP region well.



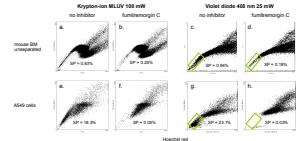
**Above.** Hoechst SP analysis of unpurified (a,c,e,g) and lineage-depleted bone marrow (b,d,f,h) on a BD LSR II flow cytometer equipped with a violet laser diode emitting at 408 nm 25 mW. Cells were incubated without (a,b) or with fumitremorgin C (c,d) prior to Hoechst 33342 labeling. The percentage SP-positive cells is indicated for each Hoechst red versus blue cytogram (a,b,c,d). Simultaneous analysis of Sca-1 versus c-kit expression for unpurified and lineage-depleted samples is shown in panels e and f (with percentage Sca-1-positive c-kit-positive cells indicated), and the Hoechst SP population for Sca-1-positive c-kit-positive fractions is shown in panels g and h.



**Above.** same as previous figure but with Power Technology VLD 401 nm laser emitting at 18 mW. Reducing the diode wavelength improved the resolution slightly but not to the level of a UV source.

## Analysis of A549 cells with UV and violet sources

A549 cells are a lung carcinoma cell line that expresses high levels of ABCG2, resulting in a high level of Hoechst SP activity. Violet laser sources CAN produce an SP population similar to UV sources for this cell type; this has led to the erroneous conclusion that violet sources are adequate for SP analysis in all cells types.



Hoechst SP analysis of unpurified bone marrow (a-d) or A549 lung carcinoma cells (e-h) on either a FACS Vantage DiVa cell sorter equipped with a krypton-ion laser emitting at 407 nm 100 mW (a,b,e,f), or a BD LSR II flow cytometer equipped with a violet laser diode emitting at 408 nm 25 mW (c,d,g,h). Cells were incubated without (a,c,e,g) or with fumitremorgin C (b,d,f,h) prior to Hoechst 33342 labeling. The percentage SP-positive cells is indicated for each Hoechst red versus blue cytogram.

## Is good Hoechst SP resolution really necessary?

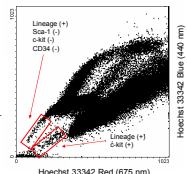
...since we still see an SP region (albeit a bad one) with violet excitation?

### Yes, it is!

Hoechst 33342 labeled bone marrow often produces more than one hypodiploid population.

We aren't sure what these low-blue populations are, but they are NOT stem cells.

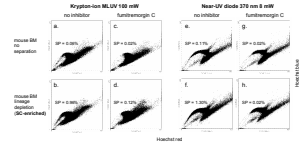
This is especially true of primate bone marrow and non-hematopoietic tissues.



**Suboptimal excitation of the Hoechst-labeled cells may fail to distinguish true SP cells from other non-stem SP populations. Violet diode excitation is not sufficient to accurately distinguish Hoechst SP cells.**

## Hoechst SP analysis with NUVLDs

When mouse bone marrow was analyzed on a BD LSR II with an NUVLD source, excellent resolution was obtained, as good or frequently better than on a stream-in-air sorter with a more powerful laser source. These small, inexpensive laser sources are therefore quite applicable for Hoechst SP when only analysis is required.



Hoechst SP analysis of unpurified (a,c,e,g) and lineage-depleted bone marrow (b,d,f,h) on either a FACS Vantage DiVa cell sorter equipped with a krypton-ion laser emitting in MLUV mode at 100 mW (a-d), or a BD LSR II flow cytometer equipped with a near-UV laser diode emitting at 370 nm 8 mW (e-h). Cells were incubated without (a,b,e,f) or with fumitremorgin C (c,d,g,h) prior to Hoechst 33342 labeling. The percentage SP-positive cells is indicated for each Hoechst red versus blue cytogram.

**Near-UV laser diodes therefore represent useful laser sources for Hoechst SP analysis. Other low-power UV sources (such as the Nd:YAG source below) should also be useful for this purpose**

